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Worse prognosis of osteosarcoma patients expressing IGF-1 on a tissue microarray

Jentzsch, Thorsten ; Robl, Bernhard ; Husmann, Maren ; Bode-Lesniewska, Beata ; Fuchs, Bruno

Abstract: BACKGROUND It is hardly possible to define osteosarcoma (OS) patients at greatest risk for non-response to chemotherapy, metastasis and short survival times. Our goal was the investigation of local expression of insulin-like growth factor (IGF-1) with regard to survival time of OS patients using a tissue microarray (TMA). MATERIALS AND METHODS Tumor tissue specimens from surgical primary tumor resections were collected from patients with OS. A TMA was composed, sections were stained with rabbit anti-IGF-1 and grading was performed. Statistics involved Kaplan-Meier curves and the log-rank test. RESULTS We analyzed immunohistochemical expression of local IGF-1 on a TMA based on surgical primary tumor resections of 67 OS patients. The mean clinical follow-up time was 98 months. Twenty-two (33%) OS patients stained negatively and 44 (66%) OS patients stained positively for IGF-1. Significantly shorter survival was detected with expression of IGF-1 ($p=0.007$). The 5-year survival rate for patients expressing IGF-1 was 63% compared to 92% in patients without expression of IGF-1. Non-responders to chemotherapy and patients with metastasis, who also stained positively for IGF-1 manifested a significantly ($p=0.002$ and $p<0.0001$, respectively) shorter survival. CONCLUSION Expression of local IGF-1 in primary tumor tissue appears to significantly affect the aggressiveness of OS, may predict survival time and, above all, may discriminate patients with non-response to chemotherapy and metastasis. This represents the basis for successful patient selection with regard to the decision process for or against chemotherapy and the choice of the most effective therapeutic drug. It may be a more important marker of tumor progression and indicator of prognosis than serum IGF-1. Novel tumor markers and therapeutic agents targeting the local IGF-1 pathway may increase the likelihood of therapeutic success.

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ZORA URL: <https://doi.org/10.5167/uzh-98588>

Journal Article

Originally published at:

Jentzsch, Thorsten; Robl, Bernhard; Husmann, Maren; Bode-Lesniewska, Beata; Fuchs, Bruno (2014). Worse prognosis of osteosarcoma patients expressing IGF-1 on a tissue microarray. *Anticancer Research*, 34(8):3881-3889.

Worse Prognosis of Osteosarcoma Patients Expressing IGF-1 on a Tissue Microarray

THORSTEN JENTZSCH^{1,2}, BERNHARD ROBL¹, MAREN HUSMANN¹,
BEATA BODE-LESNIEWSKA³ and BRUNO FUCHS¹

¹Laboratory for Orthopedic Research, Department of Orthopedics,
Balgrist University Hospital, Zürich, Switzerland;

²Division of Trauma Surgery, Department of Surgery, University Hospital Zürich, Zürich, Switzerland;

³Institute of Surgical Pathology, University Hospital Zürich, Zürich, Switzerland

Abstract. *Background:* It is hardly possible to define osteosarcoma (OS) patients at greatest risk for non-response to chemotherapy, metastasis and short survival times. Our goal was the investigation of local expression of insulin-like growth factor (IGF-1) with regard to survival time of OS patients using a tissue microarray (TMA). *Materials and Methods:* Tumor tissue specimens from surgical primary tumor resections were collected from patients with OS. A TMA was composed, sections were stained with rabbit anti-IGF-1 and grading was performed. Statistics involved Kaplan-Meier curves and the log-rank test. *Results:* We analyzed immunohistochemical expression of local IGF-1 on a TMA based on surgical primary tumor resections of 67 OS patients. The mean clinical follow-up time was 98 months. Twenty-two (33%) OS patients stained negatively and 44 (66%) OS patients stained positively for IGF-1. Significantly shorter survival was detected with expression of IGF-1 ($p=0.007$). The 5-year survival rate for patients expressing IGF-1 was 63% compared to 92% in patients without expression of IGF-1. Non-responders to chemotherapy and patients with metastasis, who also stained positively for IGF-1 manifested a significantly ($p=0.002$ and $p<0.0001$, respectively) shorter survival. *Conclusion:* Expression of local IGF-1 in primary tumor tissue appears to significantly

affect the aggressiveness of OS, may predict survival time and, above all, may discriminate patients with non-response to chemotherapy and metastasis. This represents the basis for successful patient selection with regard to the decision process for or against chemotherapy and the choice of the most effective therapeutic drug. It may be a more important marker of tumor progression and indicator of prognosis than serum IGF-1. Novel tumor markers and therapeutic agents targeting the local IGF-1 pathway may increase the likelihood of therapeutic success.

With an incidence rate approximately three to five cases *per* million population *per* year, osteosarcoma (OS) is the most common primary malignant bone tumor (1, 2). Therapeutic approaches combine neoadjuvant chemotherapy, limb-sparing surgery and histological tumor grading (3-5). Five-year survival rates usually do not exceed 75% (6, 7) and are even less favorable (20%) with non-response to chemotherapy and metastasis (8-10). Shorter survival times are also found in older patients, axial tumors and non-responders to chemotherapy (9, 10).

To facilitate the understanding of the course of disease and treatment of OS, it is crucial to detect biomarkers that predict patient survival (11). This is particularly true for rare cancers, such as OS (12-14). Only a few biomarkers (5, 15-19) for OS have been discussed. Cyr61 (5), ezrin (15), p53 (16) and tumor progression genes (17) have been associated with worse courses of disease, whereas HER2 (18, 20) has yielded equivocal results as a prognostic factor. However, they are important in order to define patient outcomes, with regard to the decision process for or against chemotherapy and the choice of drug, patient education and because survivors of sarcomas have one of the lowest health quality of life scores (21, 22).

Local expression of another biomarker, insulin-like growth factor-1 (IGF-1) has been linked to OS as well. Increased expression of IGF-1 has been shown in OS and their metastases (23, 24). But so far, local IGF-1 in primary tumor

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Abbreviations: OS: Osteosarcoma; IGF-1: insulin-like growth factor 1; IGF-1R: insulin-like growth factor 1 receptor; TMA: tissue microarray; n: number of patients; m: months.

Correspondence to: Thorsten Jentzsch, MD, Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, 8008 Zürich, Switzerland. Tel: +41 0443863095, e-mail: thorsten.jentzsch@usz.ch

Key Words: Osteosarcoma, IGF-1, TMA, chemotherapy response, metastasis, survival.

tissue has not been studied as an indicator of prognosis. IGF-1 is a hormone synthesized in the liver and to a lesser extent in autocrine/paracrine tissues. IGF-1 production is mainly stimulated by growth hormones (25). More than 98% of IGF-1 is bound to six serum proteins (26). There are four IGF-1 receptors (IGF-1R), which are tyrosine kinases (27). They require one of three ligands, namely IGF-1, IGF-2 or insulin, which bind with different affinities to their cognate and noncognate receptors. Once binding occurs, the receptor autophosphorylates tyrosine residues intracellularly (28). This activates mitogen-activated protein kinases, phosphoinositide 3-kinases and protein kinase C epsilon (29). Cell growth and inhibition of apoptosis follows (30, 31). The IGF-1 signaling pathway (32) is also responsible for osteogenesis (33), contributing to about 50% of bone cell proliferation (34).

IGF-1 has been implicated in various cancers, such as breast and prostate cancers (35-37) and congenital IGF-1 deficiency acts as a protecting factor for the development of cancer (38). Interestingly, IGF-1 has been linked to OS, because the highest levels of IGF-1 (and growth hormone) and the peak incidence of OS coincide around adolescence (23). Increased levels of IGF-1 and IGF-1R have been detected in OS (20, 24, 39). They lead to tumor progression (40) through autocrine signaling (24), angiogenesis (41), decreased susceptibility to apoptosis by *IGF-1R* overexpression (42) and metastasis (43). Increased mRNA levels of IGF-1R are also associated with proliferative OS cell line activity (44). Blockage of the IGF-1R, for example with trastuzumab (20), inhibits tumor growth of several human OS cell lines (32). Thus, IGF-1 (45) and IGF-1R (46) have been subject to various therapeutic approaches (47-49). In Ewing's sarcoma, disruption of IGF-1R signaling leads to inhibition of tumor growth and metastasis (50) by suppressing cell migration and expression of MMP-2 and MMP-9 (51). Several therapeutic approaches, such as antibodies like OncoLar (39, 52), antisense technologies (53) and dominant-negative mutants (54) have been successfully used for reduction of tumor growth and inhibition of apoptosis. Furthermore, inhibition of IGF-1R resulted in chemosensitization to conventional cytotoxic drugs, such as trastuzumab (20), doxorubicin (55), vincristine (56) and actinomycin D (57). With a few exceptions (40, 58), studies have been based on *in vitro* and *in vivo* animal experiments (25, 39), Ewing sarcomas (50, 59) and indirect assessment through OS growth inhibition by hypophysectomy (60). Furthermore, clinical investigations have not found an association between serum IGF-1 levels and patient outcomes (58, 61). So far, to the best of our knowledge there are no studies describing the local expression of IGF-1 in primary tumor tissues and its association with patient demographics and survival time in human patients (23, 24).

Currently, it is hardly possible to define OS patients at greatest risk for non-response to chemotherapy, metastasis and short survival times (19). Valuable tumor biomarkers for OS and their association with patient demographics and survival time remain barely known. Our goal was the investigation of IGF-1 with regard to demographics and survival time in OS patients using a TMA. A particular focus was put on the question whether expression of IGF-1 may identify patients with metastasis and non-response to chemotherapy, which would represent the basis for successful patient selection with regard to the decision process for or against chemotherapy, and the choice of the most effective therapeutic agent.

Materials and Methods

Patients' demographics. Tumor tissue specimens from surgical primary tumor resections were collected from bone tissue of 67 patients with OS between December 1987 and October 2005. By a retrospective chart review, a database was established containing information about patient demographics, such as response to chemotherapy, metastasis and patient survival. Tumor tissue specimens were grouped according to the histopathological classification by the World Health Organization (WHO) (62). Chemotherapy was administered according to the COSS protocol (63). According to Salzer-Kuntschik *et al.* (64), patients were responders to chemotherapy if less than 10% of vital tumor tissue was present in the surgical resection specimen. This study was conducted according to the regulations of the local ethics committee.

IGF-1 and TMA. After surgical resection of the tumor, biopsies were taken from parts with histologically-confirmed non-necrotic tumor tissues from 67 patients. A tissue microarray (TMA) (12, 19, 65-73) was set up in order to maximize the use of tumor tissue from biopsies for as many immunohistochemical tests as possible requiring only small amounts of tissue. Using a hollow needle with a diameter of 0.6 mm of a semi-automatic punch machine, between two and six tissue cores were removed from these biopsies (16, 74). Tissue cores were implanted in a recipient paraffin block in an array pattern using a computer-operated electromotor resulting in 174 spots for surgical resections. There were a total of 404 spots (Figure 1A) because several patients also provided tissue specimens from presurgical biopsies, recurrences and metastases. The paraffin block was cut into several sections of 2 µm in order to be available for immunohistochemical staining. A section of the TMA was transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA), de-paraffinized and processed with a bond automated staining system (Ventana Medical Systems, Tucson, AZ, USA). After antigen retrieval in an EDTA containing buffer (Bond Epitope Retrieval Solution 2) (Vision BioSystems, Wetzlar, Germany) for 30 min, the section was stained with a rabbit anti-IGF-1 antibody (dilution 1:50) (LabVision/Neomarkers, Fremont, CA, USA) and counterstained with hematoxylin. Visualization of primary antibody reactions, which resulted in a brown product, was undertaken by iVIEW DAB Kit (Ventana Medical Systems). Three formalin-fixed paraffin-embedded control sections with known staining patterns were used for quality control. Grading was undertaken independently by a physician, who had been trained by

Table I. Patient (number of patients=*n*) survival (mean and standard error in months=*m*) and *p*-values (log-rank test) are shown for IGF-1.

	Positive	Negative	<i>p</i> -Value
IGF-1	n=44 132±14 m 63%	n=22 198±9 m 92%	0.007

a pathologist and a student, using the MATLAB code based on color deconvolution (75, 76). In both cases, grading was carried out in a blinded fashion. In the end, more than 95% of the samples were graded similarly. For the remaining samples a consensus was found. A grading scale of 1, 2 and 3 was used. Grade 1 indicated negative (Figure 1B) staining with <10% of stained cells with no or weak staining intensity, whereas grades 2 (Figure 1C) and 3 (Figure 1D) indicated positive staining with ≥10% stained cells with weak or strong staining intensity, respectively (66). Grade 3 was reserved for staining of all cells with maximum intensity. For final evaluation purposes, we grouped negative, that is grade 1 staining, against positive, that is grade 2 and 3 staining.

Statistical analysis. Kaplan-Meier curves were used to calculate overall patient survival, which was defined as the time from diagnosis until death or last patient contact. Means and confidence intervals were stated. The log-rank (Mantel-Cox) test assessed the statistical difference between groups. Significant statistical difference was assumed if $p < 0.05$. We used the GraphPad Prism 5.01 software (GraphPad Software, La Jolla, CA, USA) and SPSS statistics v21.0 (IBM Corp., Armonk, NY, USA).

Results

Patients' demographics. We analyzed clinical parameters of 67 OS patients and immunohistochemical expression of IGF-1 on corresponding tumor tissue. Follow-up data were available for every (100%) patient and the mean clinical follow up time was 98 (range, 7 to 213) months. The overall 5-year survival rate was 73%. Our study included 24 (36%) female and 43 (64%) male patients. At the time of diagnosis, the mean age was 22 (range=2-66) years. Our study included 23 (34%) non-responders to chemotherapy and 30 (45%) responders. Chemotherapy response remained unknown in 14 (21%) patients. Nineteen (28%) patients developed metastases during follow-up, whereby 5 (7%) patients already presented with metastases at diagnosis. Twenty (30%) patients had metastases in the lung and 3 patients (4%) displayed metastases in the lung and the bone. One patient (1%) presented with metastasis at an unknown site.

Survival time and IGF-1 in a TMA. Twenty-two (33%) OS patients stained negatively and 44 (66%) OS patients stained positively for IGF-1 on the TMA. One (1%) OS patient could not be graded because spots did not contain sufficient

Table II. Patient (number of patients=*n*) survival (mean and standard error in months=*m*) in patients with OS with regard to non-response and response to neoadjuvant chemotherapy as well as expression of IGF-1.

	Non-responders		Responders		<i>p</i> -Value
	Positive	Negative	Positive	Negative	
IGF-1	n=19 85±16 m 50%100%	n=4 * 88%	n=16 174±18 m 92%	n=13 195±13 m	0.002

*No survival times were computed because all patients remained alive and were censored.

Table III. Patient (number of patients=*n*) survival (mean and standard error in months=*m*) in patients with OS with and without metastasis as well as expression of IGF-1.

	Metastasis		No metastasis		<i>p</i> -Value
	Positive	Negative	Positive	Negative	
IGF-1	n=3 50±10 m 26%	n=4 125±37 m 75%	n=24 183±13 m 92%	n=18 * 100%	<0.0001

*No survival times were computed because all patients remained alive and were censored.

material of tumor tissue. The Kaplan-Meier survival analysis showed shorter survival times for patients with immunohistochemically-detectable expression of IGF-1 in tumor tissues (Figure 2, Table I). Immunohistochemically-detectable expression of IGF-1 was indicative for shorter mean survival time of 132±14 months. In contrast, no expression of IGF-1 led to longer mean survival time of 198±9 months. When comparing these groups with a log-rank (Mantel-Cox) test, statistical significance ($p=0.007$) was reached for these two groups. The five-year survival rate of patients expressing IGF-1 was 63% compared to 92% of patients without expression of IGF-1.

Non-responders to chemotherapy who stained positively for IGF-1 manifested a significantly ($p=0.002$) shorter survival time of 85±16 months than responders to chemotherapy who stained negatively, whose mean survival time was 195±13 months (Figure 3A, Table II). The 5-year survival rate for non-responders with IGF-1 expression was 50% compared to 92% for responders without IGF-1 expression.

Interestingly, the shortest survival time of 50±10 months was found in patients with metastasis and immunohistochemical expression of IGF-1 (Figure 3B, Table III). This was significantly ($p < 0.0001$) shorter than the survival time of patients without metastasis and no immunohistochemical expression of IGF-1. No survival times were computed for

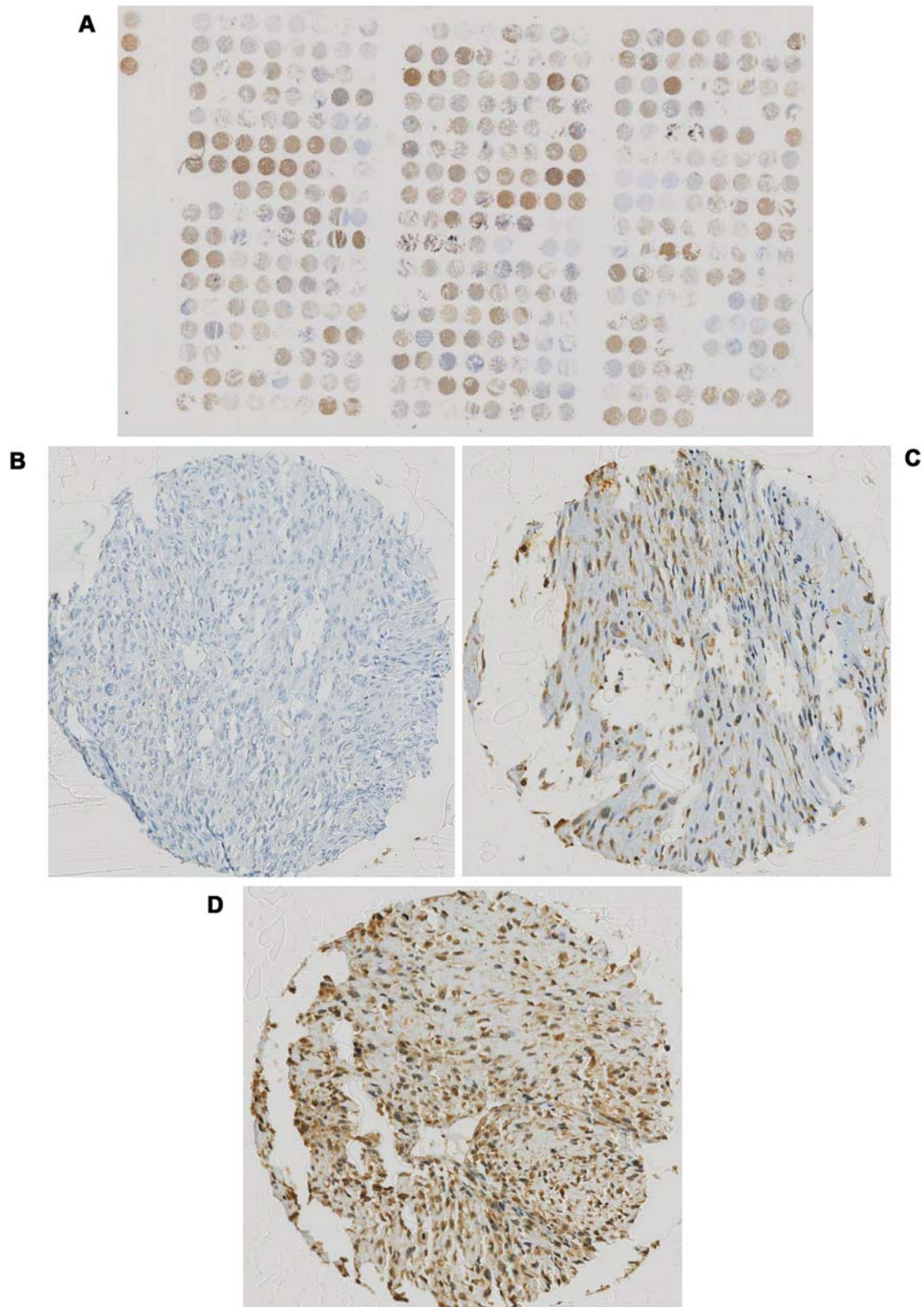


Figure 1. TMA slide with IGF-1 immunostaining. Control group of normal bone tissue on three vertical spots on the left and biopsies of OS patients on the remaining 404 spots (A). Negatively-graded spot (grade 1=B) showing mainly blue staining. Positively-graded spots (grade 2=C and grade 3=D) showing mainly brown staining.

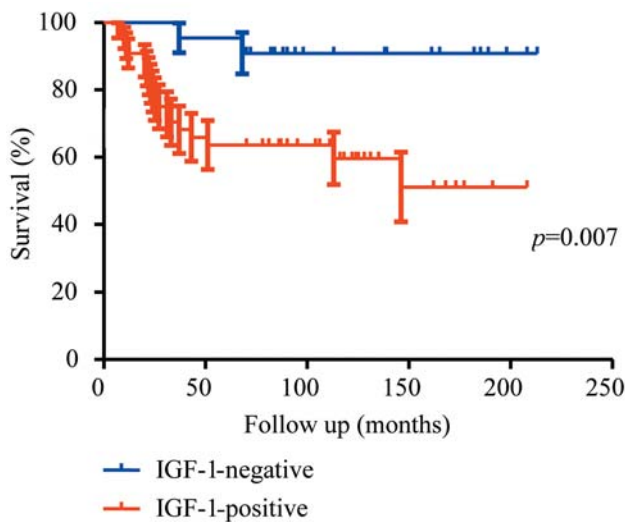


Figure 2. Patient survival in patients with OS expressing IGF-1.

patients without metastasis and no expression of IGF-1 because all patients remained alive and were censored. The 5-year survival rate for patients with metastasis expressing IGF-1 was 26% compared to 100% in patients without metastasis and no expression of IGF-1.

Discussion

Our study suggests that local IGF-1 expression in surgical primary tumor resection tissue specimens in patients with OS goes along with more aggressive tumor types associate with an increased number of metastasis, non-response to chemotherapy and worse survival time.

OS is the most common primary malignant bone tumor (1), but relatively rare in comparison to other cancers (35, 36). Survivors of sarcomas have one of the lowest health quality of life scores (22). Particularly, valuable tumor biomarkers for OS and their association with patient demographics and survival time remain barely studied because patient data are limited (19). However, they are especially important in order to define patient outcomes at the time of diagnosis, treatment decisions and ultimately patient education (21). The role of IGF-1 in OS has been investigated in several studies (20, 23, 24, 58, 61), but to the best of our knowledge no clinical information is available on the expression of local IGF-1 of the primary tumor as an indicator of prognosis (23, 24). Burrow *et al.* (24) examined 48 patients with osteosarcoma with regard to their IGF-1 expression in primary tumors and metastasis using reverse transcriptase-polymerase chain reaction (RT-PCR). Twenty-seven (61%) of 44 tumors expressed levels of IGF-1 greater

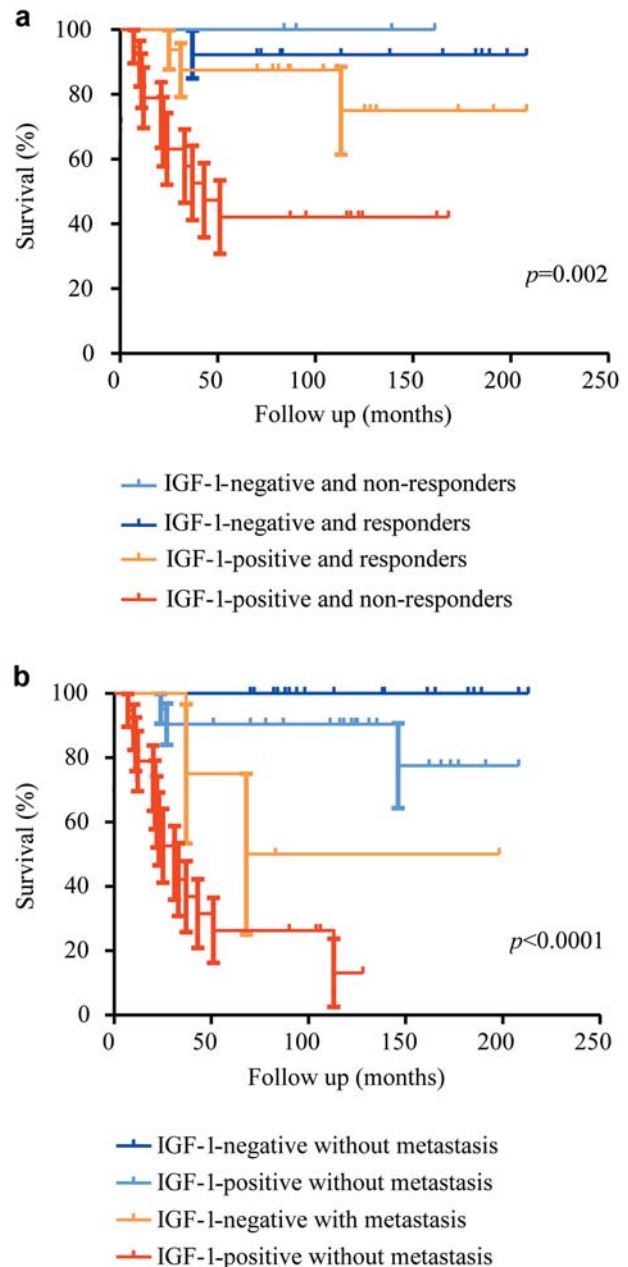


Figure 3. Patient survival in patients with OS in non-responders and responders to chemotherapy and IGF-1 expression (A) as well as with and without metastasis and expression of IGF-1 (B).

than or equal to the positive control group. Higher levels of IGF-1 were not correlated with metastasis. However, they did not correlate their findings with clinical patient data. Another study by Scotlandi *et al.* (20) investigated the IGF-1R functions in two Ewing sarcoma and two OS cell lines. They studied the *in vitro* efficacy of trastuzumab in association with anti-IGF-1R treatment (neutralizing anti-IGF-1R α IR3

monoclonal antibody) because IGF-1R plays a role in resistance to trastuzumab (20, 37). Their results showed that α IR3 enhanced the trastuzumab-induced growth inhibition in these cell lines. This was confirmed by an increased susceptibility to trastuzumab in Ewing sarcoma cell lines, TC-71 derived clones with impaired IGF-1R functions (20).

Above all, IGF-1 discriminates patients with metastasis and non-response to chemotherapy. This represents the basis for successful patient selection with regard to the decision process for or against chemotherapy and the choice of the most effective therapeutic drug. In the future, it may be advisable to separate patients according to the expression of IGF-1 and possibly treat only those who are most likely to benefit from chemotherapy. Thus, patients who are not likely to benefit from chemotherapy may not have to undergo unnecessary chemotherapy that is accompanied by many adverse effects.

Our study adds important information to the current literature, because we point out that local IGF-1 expression in primary tumor tissue may be a more important indicator of tumor progression and of prognosis than serum IGF-1. Circulating concentrations of IGF-1 were previously described as non-predictive of clinical characteristics of OS patients in a study by Rodriguez-Galindo *et al.* (61). They investigated serum IGF-1 in 37 patients with OS by ELISA. IGF-1 levels were not significantly associated with response to chemotherapy ($p=0.95$), metastasis ($p=0.12$) and survival time ($p=0.52$). Likewise, Borinstein *et al.* (58) recently investigated serum levels of IGF-1 in 142 OS patients and did not find an association with overall survival. In a murine OS model, Hong *et al.* (23) did not find evidence that serum IGF-1 played a role in local tumor progression or metastasis. They injected K7M2 murine OS cells into genetically-engineered, liver-specific IGF-1-deficient mice with 75% reduction in IGF-1 levels while maintaining IGF-1 in the tumor environment. On the other hand, there are reports about increased IGF-1 levels leading to tumor growth (40) through autocrine signaling (24), angiogenesis (41), decreased susceptibility to apoptosis by IGF-1R overexpression (42) and metastasis (43). Furthermore, Kolb *et al.* (39) reported inhibition of tumor growth by anti-IGF-1R antibodies in xenografts *in vivo*. Other pre-clinical data suggest efficacy of IGF-1R antagonists (47) and these results were investigated in a recent phase-2 study by Weigel *et al.* (48). They studied the efficacy, pharmacodynamics and toxicities of single-agent cixutumumab, which is a human IgG1/ λ monoclonal antibody against IGF-1R leading to inhibition of the IGF pathway (49) in solid tumors including OS. Administration of cixutumumab was tolerated well and led to increased serum levels of IGF-1, confirming IGF-1 as an important biomarker in OSs. In other words, our study points-out that local IGF-1 expression in primary tumor tissue may be

much more important in tumor progression and as an indicator of prognosis than serum IGF-1. In the future, novel tumor markers for the IGF-1 pathway that target local IGF-1 may increase the likelihood of therapeutic success.

To date, there are only a few OS TMAs in the commonly cited in the English literature (12, 19, 67-73, 77). Patient numbers are rather small, clinical information is limited and tissue specimens usually derive from presurgical biopsies. As a possible limitation, our study exclusively investigated primary tumor tissue resections after chemotherapy instead of biopsies before chemotherapy. Nonetheless, an additional predictor of survival, aside from tumor necrosis, is very important for clinicians and patients alike; no matter whether this particular discriminator stems from the pre- or post-chemotherapeutic phase. Furthermore, decisions on adjuvant chemotherapy are not only based on biopsies, but also on tumor necrosis rate in primary tumor tissue resections. Therefore, IGF-1 in primary tumor tissue resections provides an additional discriminator for patients at an increased risk for shorter survival time, which may arise from non-response to chemotherapy or metastasis. Furthermore, immunohistochemistry of a TMA has limited power in terms of antigen quantification. Nonetheless, the advantage of a TMA is the evaluation of the local expression of different tumor biomarkers in a large sample number under the same conditions (65). It is understood that the leading value of this study lies in its novelty report of worse prognosis of osteosarcoma patients expressing local IGF-1 in primary tumor tissue on a TMA by combining clinical patient data with local tumor biomarker expression in an immunohistochemical test method.

Conclusion

Our study suggests that local IGF-1 expression in surgical primary tumor resection tissue specimens of patients with OS is associated with more aggressive tumor types and survival time. Above all, IGF-1 discriminates patients with metastasis and non-response to chemotherapy. This represents the basis for successful patient selection with regard to the decision process for or against chemotherapy and the choice of the most effective therapeutic drug. Furthermore, we point-out that local IGF-1 expression in primary tumor tissues may be much more important in tumor progression and as an indicator of prognosis than serum IGF-1. In the future, novel tumor markers and therapeutic agents for the IGF-1 pathway that target local IGF-1 may increase the likelihood of therapeutic success.

Conflicts of Interest

The Authors declare that they have no competing interests.

Acknowledgements

We would like to thank Ms. Chantal Pauli, MD, for her training in TMA grading, Ms. Martina Storz-Schweizer for her help with TMA management as well as Mr. Yufei Li, MSc, and Mr. Roman Muff, PhD for the development of the TMA MATLAB tool.

Our work is supported by the University of Zürich, the Schweizerischer Verein Balgrist (Zürich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zürich, Switzerland), the Highly Specialized Medicine for Musculoskeletal Oncology program of the Canton of Zürich, the Zürcher Krebsliga (Zürich, Switzerland), and the Swiss National Science Foundation SNF Nr.310030_149649. The funders had no role in the preparation of the manuscript.

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Received May 27, 2014

Revised June 18, 2014

Accepted June 19, 2014